Integrating Morphological and Molecular Data for Resolving Lucinidae (Bivalvia) Phylogenies: Implications for Taxonomy and Fossil Inclusion

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Abstract

Lucinidae, an ancient clade of chemosymbiotic bivalves dating back to the Late Jurassic, have undergone changing taxonomic classifications. Older morphology-based classifications conflict with recent molecular phylogenies. Current taxonomies rely on molecular data, limiting phylogenetic placement to extant taxa with available molecular data. To better understand lucinid evolutionary history, a phylogenetic hypothesis including fossil taxa and morphological characters is needed. Here, morphological and molecular character data are examined using species-level phylogenetic analyses of 52 Neogene and Quaternary lucinid taxa from the Western Atlantic. A morphological matrix of 58 shell characters was developed to describe interior and exterior shell features, including ornamentation, hinge and dentition, muscle scars, pallial line, and inhalant channel, a feature inferred to be associated with chemosymbiosis in lucinids. Published molecular data included two nuclear ribosomal genes (18S and 28S rRNA) and the mitochondrial cytochrome *b* gene for 18 extant species. We examine congruence and resolution in cladograms produced using 1) parsimony and Bayesian inference methods, 2) morphological characters and combined morphological-molecular characters, and 3) pruned morphology-only, combined morphological-molecular cladograms, a reanalyzed molecular-only tree, and a pruned previously published tree for the family. Bayesian cladograms based on morphological and combined morphological-molecular data were better resolved than those from parsimony methods. While morphological trees had poor resolution at deeper nodes and were uninformative for subfamily-level designations, they successfully placed species into genera and aligned with molecular phylogenies at the tips. Combining molecular data with morphological characters improved resolution at deeper nodes and increased congruence with published phylogenies. Thus, integrating both data types provided clearer species-level placement than morphology alone. Recent phylogenetic studies often overlook morphological characters in place of molecular data, however, this study indicates that the combined use of morphological and molecular characters allows the generic-level placement of fossil taxa and living taxa that do not have molecular data.

Keywords
Phylogenetic; Morphological; Bivalvia; Lucinidae

Introduction

The Lucinidae are the most speciose family of chemosymbiotic bivalves with extant members widely distributed geographically (60 N to 55 S), bathymetrically, and ecologically (intertidal mangrove forests to hydrocarbon vents) (Taylor and Glover 2006, 2010). The family has particularly high diversity in seagrass biomes (Stanley, 2014; Taylor and Glover, 2022). Lucinids also have a long evolutionary history; the Ordovician *Babinka* possesses diagnostic features of lucinids (Taylor and Glover 2022) as does the Silurian, *Illionia prisca*, which is found preserved *in situ* in a life position characteristic of most living members of the family (i.e., anterior-posterior axis parallel to the sediment-water interface) (Liljedahl 1992). Based on possession of characters diagnostic of lucinids, it is inferred that chemosymbiosis was in place by at least the Silurian although independent and corroborating sedimentologic/diagenetic and geochemical evidence dates only to the Late Jurassic (Gaillard et al. 1992; Peckmann et al., 1999).

The early taxonomies and phylogenies of lucinids, such as those by Chavan (1969) and Bretsky (1970, 1976), provided foundational classifications but are inconsistent with recent molecular phylogenies. Chavan's taxonomy, which grouped genera into four subfamilies without detailed criteria, contrasts with Bretsky's stratigraphic reconstruction of morphologically similar taxa based on a phenetic analysis. However, even Bretsky's results from 1970 and 1976 do not align, highlighting challenges in resolving lucinid evolutionary relationships due to homoplasy and convergence at the generic level. Both Chavan and Bretsky noted uncertainties regarding the monophyly of lucinids with divaricate sculpture, which often show analogies with nondivaricate taxa. These inconsistencies, summarized in Table 1, underscore the limitations of morphology-based approaches and the need for molecular data to resolve phylogenetic relationships.

Because recent lucinid phylogenies exclusively use molecular sequence data (Williams et al. 2004; Taylor and Glover 2006; Taylor et al. 2011, 2016, 2022a, 2022b), fossil taxa cannot be incorporated to fully document lucinid evolutionary history. In addition, the incorporation of morphologic data from living and/or fossil taxa has been found in some cases to increase congruence (Stockley et al. 2005; Legg et al. 2013; Thy and Stohr 2016; Mongiardino Koch et al. 2021; Asher and Smith 2022), increase resolution and support (Heikkila et al. 2014), reveal key morphologic synapomorphies (Stockley et al.2005; Bieler et al. 2014) and improve the ability to distinguish among models of quantitative trait evolution (Slater et al. 2012). Alternatively, incorporating data (molecular and morphology) from living taxa, can improve accuracy in studies focused on fossil taxa (e.g., Wiens 2009; Asher and Smith 2022).

Table 1: Comparison of lucinid classifications and phylogenetic approaches.

Study	**Contribution**	**Key Findings**	**Notes on Consistency with Modern Phylogenies**
Chavan (1969)	Established the first comprehensive taxonomy of recent and fossil lucinids.	Divided genera into four subfamilies.	Few classification criteria provided; largely inconsistent with modern molecular phylogenies.
Bretsky (1970)	Conducted a phenetic analysis to explore lucinid evolutionary relationships.	Proposed a phylogeny of North American genera and subgenera.	Results reflect strato-morphologic approaches; inconsistencies due to homoplasy in morphological traits.
Bretsky (1976)	Produced the first quantitative reconstruction of lucinid evolutionary relationships.	Built on earlier phenetic analysis to refine phylogeny.	Highlights the limitations of morphology-only approaches for deeper evolutionary nodes.
Taylor and Glover (2006)	Re-evaluated morphological and molecular data in lucinid phylogeny.	Discussed homoplasy in morphological characters and its impact on phylogenetic reconstruction.	Helped align molecular and morphological phylogenies, but inconsistencies remain.
Taylor et al. (2011, 2014, 2016)	Advanced molecular phylogenetic analyses.	Provided a clearer framework for evolutionary relationships using molecular markers.	Modern phylogenies diverge from earlier morphology-based classifications.

For lucinids, identifying morphologic synapomorphies for molecularly defined subfamilies and lower taxa is straightforward for some taxa (e.g., Codakiinae, Pegophyseminae) and in other cases is not (e.g., Leucospharinae) (Taylor and Glover 2005, 2006, 2016; Taylor et al. 2011, 2022; Williams et al. 2020). In other groups, however, even when homoplasy is high, morphology may still contain phylogenetic signal (Amor et al. 2016; Long-Fox 2022), can still contribute to well-supported relationships (Bieler et al. 2014; da Silva Paiva 2020) and be diagnostic at lower taxonomic levels (Savenko et al. 2021). In a combined morphological and molecular analysis for major bivalve lineages, shell characters were found to be phylogenetically informative (Bieler et al. 2014)

This study presents a new morphological character matrix, which modified and expanded on previous work (Bretsky 1970, 1976). Here, phylogenetic trees based on this new morphological character matrix are presented, and congruence between and resolution differences among morphologic, molecular, and combined trees are described for the following analyses:

1. Bayesian inference and parsimony methods to determine which model, using morphological data, provided greater resolution and congruence with current molecular trees (Taylor et al. 2011, 2016);
2. Combined morphological characters and molecular data for published sequence data for three genes (18S rRNA, 28S rRNA, and cyt *b*) that produce congruence among phylogenetic trees using only morphological characters and those integrating morphological and molecular data;
3. Comparing Bayesian phylogenetic trees from this study with the combined gene phylogenetic tree of Taylor et al. (2016) as an exemplar molecular phylogeny. Direct

comparisons were made between a pruned molecular-only tree (Taylor et al. 2016), a pruned Bayesian combined morphological character and molecular tree, a pruned Bayesian morphological character-only tree, and a molecular tree using only western Atlantic taxa.

Materials and Methods

Taxa Selection

A species-level analysis of 52 Lucinidae ingroup taxa was conducted to compare the phylogenetic positions of extinct and extant taxa using morphological and molecular characters. Ingroup lucinid taxa from a range of depths and habitats were selected based on spatial (Western Atlantic) and temporal (Neogene to the Present) distributions determined from the literature, World Register of Marine Species (WoRMS), and The Paleobiology Database (PBDB). The selected ingroup lucinid taxa are limited in both spatial and temporal distributions to provide a comparable taxonomic representation to previous morphological phylogenies (Bretsky 1970, 1976; Christie et al. 2016; Christie 2017) and allow focus on morphological versus molecular differences at a higher taxonomic resolution than previous full-scale family-level molecular phylogenies (Williams et al. 2004; Taylor and Glover 2006; Taylor et al. 2011, 2016). Two Thyasiridae taxa, *Parathyasira equalis* and *Thyasiria biplicata*, were selected as outgroup taxa because morphological and molecular evidence places thyasirids as the sister group to lucinids (Bieler et al. 2014). Thyasirids share similar shell morphology with lucinids, and some species or individuals house endosymbionts, although their presence can be absent or facultative for some species (Dufour 2005). Like lucinids, thyasirids have widespread habitat and geographic ranges, with *P. equalis* and *T. biplicata* occuring throughout the northern Atlantic (Taylor et al. 2007;

Duperron et al. 2013). Information on all analyzed taxa, including photographs, temporal range, material examined, and papers referenced are listed in Supplemental Material.

Morphological Data

Fifty-eight morphological characters were developed to describe interior and exterior shell features including ornamentation, hinge and dentition, muscle scars, pallial line, and inhalant channel (Supplemental Material). This suite of characters and character states were either newly developed for this study (n=24) or modified from Bretsky (1970) (n=34). For each taxon examined, morphological character coding was performed using type specimens or their images (as available) as exemplars, in conjunction with non-type specimens obtained from additional localities (Listed in Supplemental Material). The 54-species and 58-morphological character matrix was stored as a NEXUS file (Maddison et al. 1997) in Mesquite (Maddison and Maddison 2018) for phylogenetic analyses and is provided in the Supplemental Material (File S1) and on MorphoBank (O'Leary and Kaufman 2011, 2012) as P4896 (http://morphobank.org/permalink/?P4896).

Molecular Data

Published molecular sequences for two nuclear ribosomal genes (18S and 28S rRNA) and the mitochondrial gene cytochrome *b* (cyt *b*) from Taylor et al. (2011, 2016) were downloaded from GenBank (Benson et al. 2013; Sayers et al. 2020, 2021) for 19 lucinid ingroup species and both thyasirid outgroup species (Table 2). For taxa in this study, only specimens with at least two of the three gene sequences were used. Two ingroup taxa (*Epicodakia pectinata* and *Lucina aurantia*) were not included in the molecular data analyses because they only had cyt *b* sequence data. Sequences were aligned using Clustal Mega 7 (Kumar et al. 2016), following the parameters found in Taylor et al. (2011) with gap opening penalty set to 15, gap extension

penalty set to 7, and delay divergent cutoff percent set to 95%. Poorly aligned regions and gaps were removed from sequences using Gblocks server version 0.91b (Castresana 2000; Talavera and Castresana 2007), following settings given in Taylor et al. (2016) for less stringent selection that allows gaps within final blocks and less strict flanking positions. After sequences were analyzed in Gblocks, 18S rRNA gene was reduced from 1,783 bp to 942 basepairs (bp) (representing 52% of the original data), 28S rRNA gene was reduced from 1,669 bp to 1,379 bp (representing 82% of the original data), and 100% of the original data remained for the cytochrome *b* gene (355 bp). These sequence lengths were comparable to those in Taylor et al. (2011, 2016). The aligned molecular sequence data were exported as NEXUS files (Maddison et al. 1997) and concatenated for phylogenetic analyses (File S2).

Datasets

Three datasets were compiled: (1) a morphological dataset (File S1) including all taxa (n = 54) for morphology-only phylogenetic analyses; (2) a molecular dataset (File S2) including taxa with molecular data (n = 21), used to compare results with a pruned molecular tree from Taylor et al. (2016); and (3) a combined dataset (File S3) integrating morphological and molecular data for all taxa (n = 54), concatenating three genes (18S rRNA, 28S rRNA, and cyt b) to investigate the effects of combining these data.

Parsimony Phylogenetic Analyses

Parsimony analyses were performed on the morphological dataset (File S1) and the concatenated morphological and molecular dataset (Files S3) using the software package PAUP: Phylogenetic Analysis Using Parsimony (PAUP*) version 4.0a (Swofford 2002). Using a heuristic search algorithm with the starting trees for branch-swapping by random stepwise addition (settings: swap only the best, number of trees at each step kept = 5, repetitions = 10,

seed = 0, hold 1 tree at each step). Branch swapping was set to tree bisection-reconnection (TBR) and optimizing unordered (Fitch) characters was set to accelerated transformation (ACCTRAN). All transformation costs were equal. Branch support was assessed with a bootstrap resampling method (Felsenstein 1985) performed with 100 replicates. For each dataset, a majority-rule consensus tree was generated for all bootstrap trees with over 50% node support.

Bayesian Phylogenetic Analyses

Bayesian phylogenetic analyses were conducted using Markov chain Monte Carlo (MCMC) methods (Metropolis et al. 1953; Hastings 1970) in MrBayes version 3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003; Ronquist et al 2012) on all three datasets (Files S1, S2, and S3). Bayesian settings followed those listed in Williams et al. (2004) and Taylor et al. (2011, 2016). Each dataset was run for 50,000,000 generations, sampling trees every 5,000 generations, with the first 25% discarded. Previous studies determined a General Time Reversible substitution model (GTR) with a proportion of invariable sites (I) and a gamma shaped distribution of rates across sites (Γ), or a GTR + I + Γ, was the best model for these molecular datasets (Williams et al. 2004; Taylor et al. 2011, 2016). Therefore, for molecular data, the GTR + I + Γ nucleotide substitution model was selected using the following settings: rates=invgamma, nst=6. The default substitution model, a gamma-shaped rate variation with all substitution rates equal, was used for morphological data. For this model settings were: rates=gamma, nst=1. For combined morphological and molecular datasets, data were partitioned (set partition = favored) with molecular data assigned to run under a GTR + I + Γ substitution model and morphological data assigned to run under a gamma-shaped rate variation model. For each Bayesian phylogenetic analysis, a 50% majority rule consensus tree was calculated with posterior probabilities.

193 **Table 2:** Taxa included in analyses with the associated locality, museum/reference collection source materials (specimen voucher),
194 and the GenBank accession numbers for the nuclear 18S and 28S ribosomal RNA genes and the mitochondrial gene cytochrome *b*.

Taxa	Locality	Museum/Reference	18S rRNA	28S rRNA	Cyt *b*
Anodontia alba	^ Guadeloupe	IM-2013-20174	LT614694	LT614737	LT614772
Cavilinga blanda	^ Guadeloupe	IM-2013-8163	LT614696	LT614739	LT614774
Clathrolucina costata	* Bocas, Panama	BMNH 20100252	FR686727	FR686809	FR686628
Codakia orbicularis	* Little Duck Key, FL	BMNH 20100281	AM774500	AM779674	FR686625
Ctena imbricatula	* Bocas, Panama	BMNH 20100263	FR686715	FR686829	FR686636
Ctena orbiculata	^ West Summerland Key, FL	NHMUK 20160350	LT614691	LT614735	LT614770
Divalinga quadrisulcata	* Guadeloupe	-	AJ581854	AJ581888	FR686644
Divalinga weberi	^ Bocas, Panama	NHMUK 20160343	LT614690	LT614734	LT614768
Ferrocina garciai	^ LA	USNM 1227857	-	KF793276	KF793275
Lucina pensylvanica	* Lower Matecumbe Key, FL	BMNH 20070311	AM774127	AM774138	AM774148
Lucina roquesana	*^ Los Roquas, Venezuela	BMNH 20100282	FR686738	FR686805	FR686659
Lucinisca nassula	* Little Duck Key, FL	BMNH 20100245	FR686736	FR686812	FR686657
Lucinisca muricata	^ Guadeloupe	IM-2013-9474	LT614703	LT614745	LT614779
Mytrina pristiphora	^ Guadeloupe	IM-2013-9474	LT614705	LT614747	LT614781
Parvilucina crenella	* Ramrod Key, FL	BMNH 20100273	FR686741	FR686799	FR686669
Parvilucina pectinella	^ Guadeloupe	IM-2013-6577	LT614708	LT614750	LT614784
Phacoides pectinatus	*Fort Pierce, FL	BMNH 20070291	AM774503	AM779677	FR686674
Radiolucina amianta	*Ramrod Key, FL	BMNH 20100247	FR686745	FR686813	FR686676
Stewartia floridana	*Cedar Key, FL	BMNH 20100260	FR686749	FR686797	FR686684
Outgroups:					
Parathyasira equalis	* Gullmarsfjorden, Sweden	BMNH 20070296	AM392453	AM392437	FR686685
Thyasira polygona †	* Northern North Sea	BMNH 20070298	AM774484	AM392433	FR686686

195 * Denotes used in Taylor et al. 2011
196 ^ Denotes used in Taylor et al. 2016
197 *^ Revised species name originally in Taylor et al. 2011 then changed in Taylor et al. 2016
198 † *Thyasira polygona* (Jeffreys, 1864) is synonymized with *T. biplicata* (Philippi, 1836) at the National Museum of Wales

Trees

Trees were visualized and branches were rotated in FigTree v1.4.3 (Rambaut 2018) and Mesquite (Maddison and Maddison 2018). The trees are cladograms that depict topology only, with branch lengths carrying no specific meaning. Bayesian trees from the morphological-only and combined morphological and molecular datasets were pruned to be directly comparable with the combined gene phylogenetic trees from Taylor et al. (2016) as well as a molecular only tree produced from the 18 ingroup taxa used in this study.

Unlike molecular datasets, morphological datasets lack statistical methods for model selection, complicating the choice between probabilistic (Bayesian) and parsimony-based approaches. Parsimony methods produce trees by minimizing evolutionary steps, with the shortest tree length preferred (Wheeler 2012). Bayesian methods, by contrast, select trees with maximum posterior probability, reflecting the highest likelihood given the data, model, and edge probabilities (Wheeler 2012; Baum and Smith 2013).

Results

Morphological Phylogenetic Analyses

For the morphological dataset both Bayesian and parsimony analyses produced low-resolution trees that consisted of a large basal polytomy and relatively few defined clades toward the tips (Figures 1 and 2). The Bayesian analysis of the 54-species and 58-morphological character matrix resulted in a consensus tree with 18 nodes (Figure 1). Posterior probabilities ranged from 55 to 100%, with twelve node above 70% and seven nodes between 55 and 60% (Figure 1). The parsimony analysis using a heuristic search resulted in a consensus tree with 12 nodes, all with 100% bootstrap support (Figure 2). Despite weak node support (posterior probability of 58% and 59%) in the Bayesian consensus tree, strong bootstrap support was

observed for the *Ctena imbricatula* and *C. orbiculata* pair and the *Eomiltha pandata* and *E. scolaroi* pair. In the *Lucina* clade, the Bayesian consensus tree supported a sister relationship between *L. aurantia* and *L. pensylvanica/L. roquesana*, equating them with *L. glenni*. All other node comparisons between Bayesian posterior probabilities and parsimony bootstrap support were consistently well-supported in both analyses. In both analyses, the following clades were recovered: thyasirid outgroup, *Ctena*, *Cavilinga*/*Clathrolucina*, *Divalinga*/*Divaricella* (without *Divaricella dentata*), *Lucina*, *L. pensylvanica*/*L. roquesana*, *Stewartia*, *Eomiltha*, and *Miltha*/*Armimiltha* (Figures 1 and 2). The Bayesian analysis was also able to resolve the following species pairs: *Epicodakia filiata* and *E. pectinata*, *Ferrocina cubana* and *F. garcai*, and *Radiolucina amianta* and *R. waccamawensis* (Figure 1). In addition, there was greater resolution for the *Lucina* clade in the Bayesian analysis (Figure 1). Further, three *Pleurolucina* species were basal within the *Lucina* clade in the Bayesian analysis (Figure 1), but were each part of the basal polytomy in the parsimony analysis (Figure 2).

The only unique character state was character number 35, describing an absent or obscure escutcheon in lucinids and its presence in the outgroup taxa, *Thyasira polygona* and *Parathyasira equalis*. Inspection of characters present in clades indicated that character number 19, describing the posterior ventral notch, was prominent only within the *Lucina* clade and was present within most *Pleurolucina* (the exception being *P. hendersoni* with a shallow posterior ventral notch). Of the clades present, *Divalinga weberi* had numerous missing character states, but was still accurately placed with *Divalinga quadrisulcata* and *Divaricella chipolana*. Characters 14 and 15 (periostracum and periostracum color, respectively) were missing for many taxa and, because of the frequently unknown state of these characters, were not useful in this phylogenetic study. Further, for characters that build upon other characters (i.e., rib descriptions),

245 missing data were generated when a given character was not applicable for certain taxa,
246 including character 22 (sharpness of dominate surface sculpture), 23 (commarginal rib spacing
247 relative to interspaces), 24 (radial rib spacing relative to interspaces), 25 (radial rib bifurcation),
248 and 26 (spinosity of radial ribs). However, these sculpture and rib characters were highly
249 valuable for the morphological analysis, as they helped resolve the genera *Radiolucina*,
250 *Ferrocina*, *Ctena*, and *Epicodakia* due to their distinctive shell ornamentation.

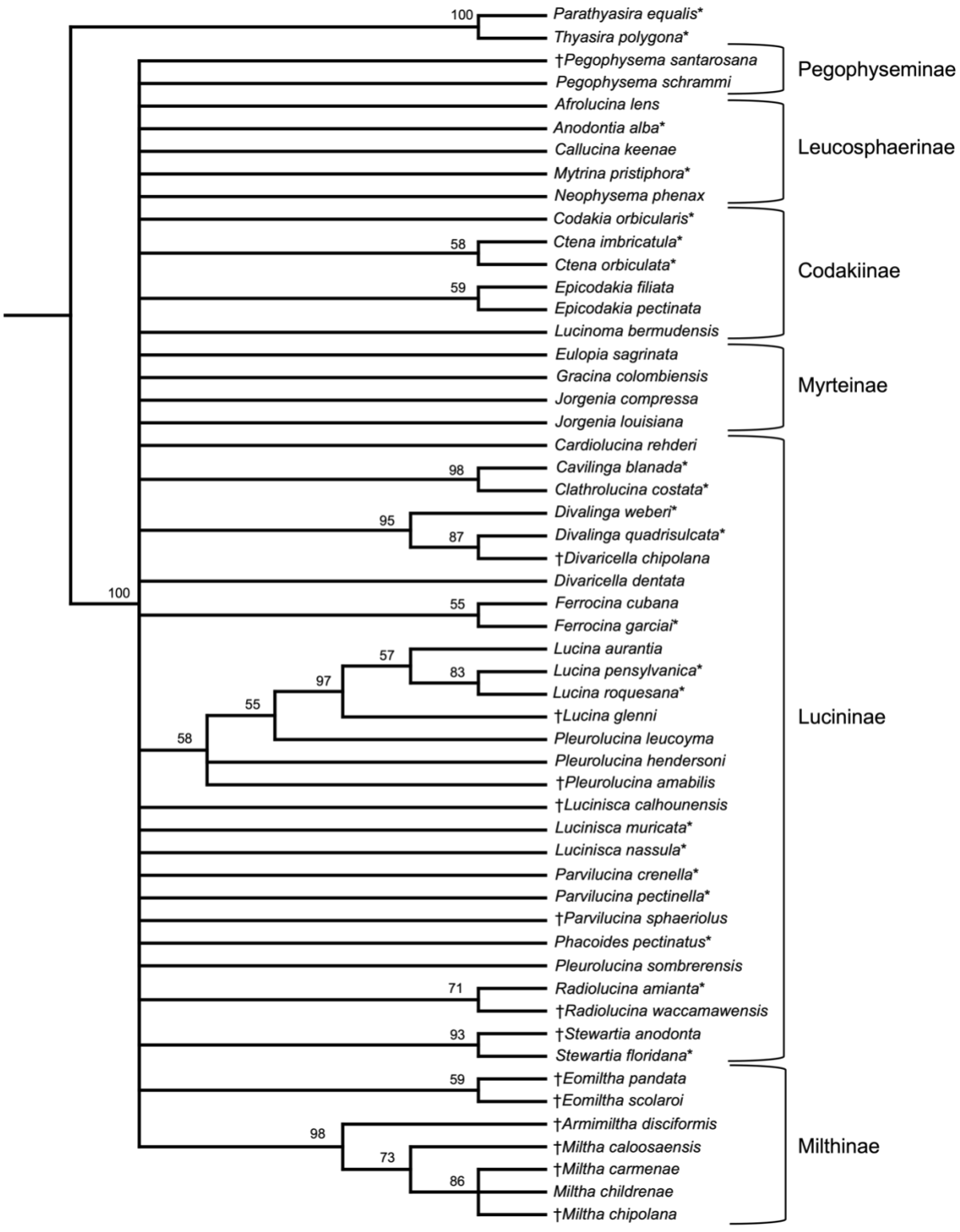

Figure 1: Morphological cladogram of extant and fossil taxa produced using Bayesian analysis. Support values at nodes are posterior probabilities. Subfamilies designated in Taylor et al. (2011; 2016). Asterisks indicate molecular data is available for that species; dagger indicates that a species is extinct.

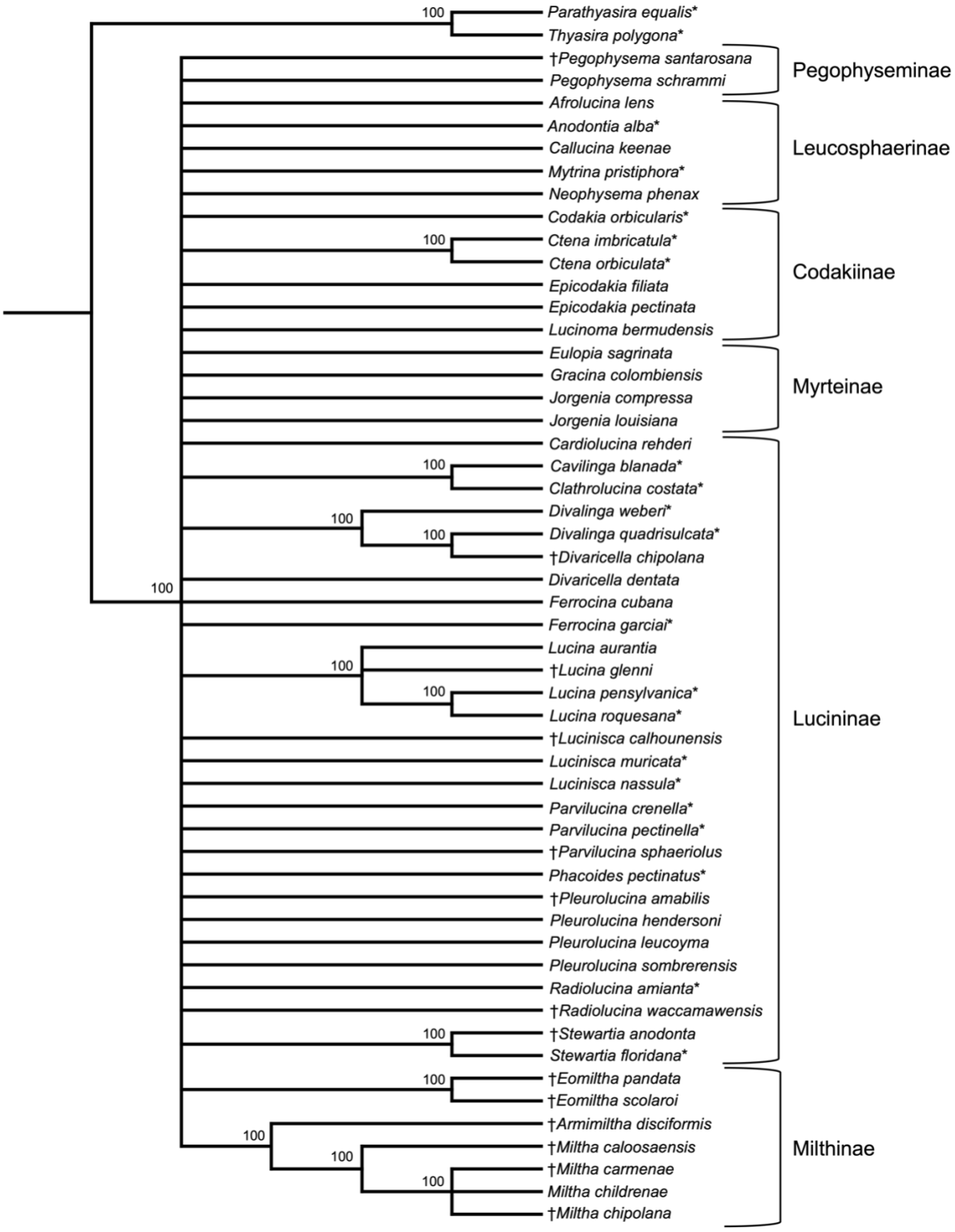

Figure 2: Morphological cladogram of extant and fossil taxa produced using parsimony analysis. Support values at nodes are bootstrap. Subfamilies designated in Taylor et al. (2011; 2016). Asterisks indicate molecular data is available for that species; dagger indicates that a species is extinct.

Combined Morphological and Molecular Data

The combined morphological and molecular dataset produced trees (Figures 3 and 4) that were more resolved than the morphology-only trees (Figures 1 and 2). The Bayesian analysis of the 58 morphological characters and 2,679 molecular characters yielded a consensus tree with 21 nodes (Figure 3). The parsimony analysis, using a heuristic search of the 2,737-character matrix, generated a consensus tree with 15 nodes, including 1,928 constant characters (70.4%), 233 variable parsimony-uninformative characters, and 576 parsimony-informative characters (Figure 4).

As with morphology-only trees, the parsimony tree was less resolved than the Bayesian tree, all nodes in the parsimony analysis had 100% bootstrap support, and the Bayesian tree had low (53 - 99%) posterior probability values (Figures 3 and 4). In both analyses the following clades were recovered: 1) thyasirid outgroup, 2) *Ctena/Codakia*, 3) *Divalinga*/*Divaricella* (without *D. dentata*), 4) *Lucina*, 5) *Lucinisca*, 6) *Radiolucina*, 7) *Stewartia*, 8) *Eomiltha*, and 9) *Miltha* (without *M. caloosaensis*) (Figures 3 and 4). In contrast, the parsimony tree included two additional taxa and greater resolution within the subfamily Milthinae than in the Bayesian tree (Figures 3 and 4). Alternatively, the Bayesian analysis resolved more species into paired clades that included: 1) *Epicodakia filiata*, *E. pectinata*, *Codakia orbicularis*, and *Ctena imbricatula*; 2) *Cavilinga* within a clade of *Lucina* and *Divalinga* and *Divaricella*; 3) *Parvilucina* group, and 4) *Pleurolucina* group (Figure 4).

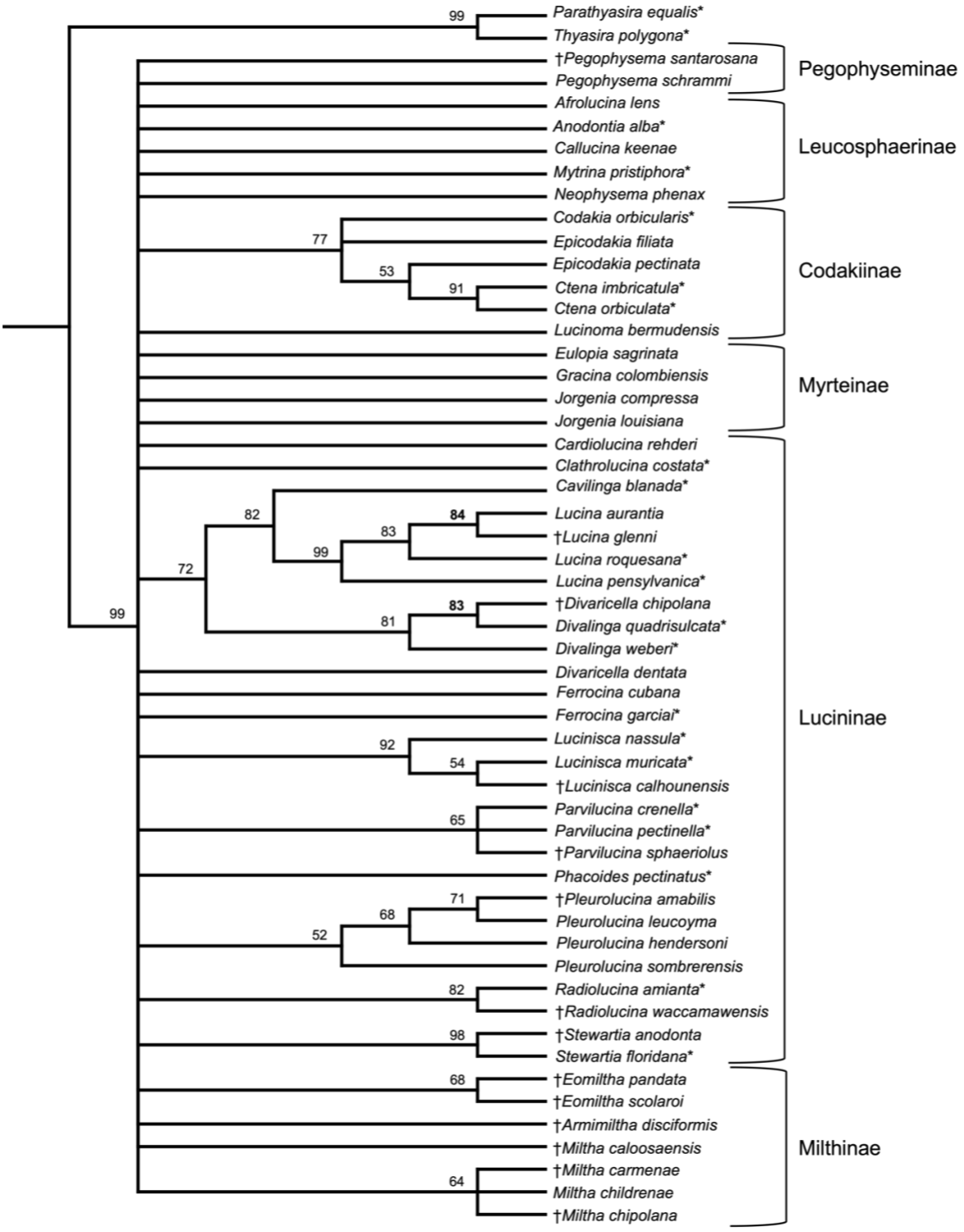

Figure 3: Combined morphological and molecular (concatenated dataset of 18S rRNA, 28S rRNA, and cyt *b*) cladogram of extant and fossil taxa produced using Bayesian analysis. Support values at nodes are posterior probabilities. Subfamilies designated in Taylor et al. (2011; 2016). Asterisks indicate molecular data is available for that species; dagger indicates that a species is extinct.

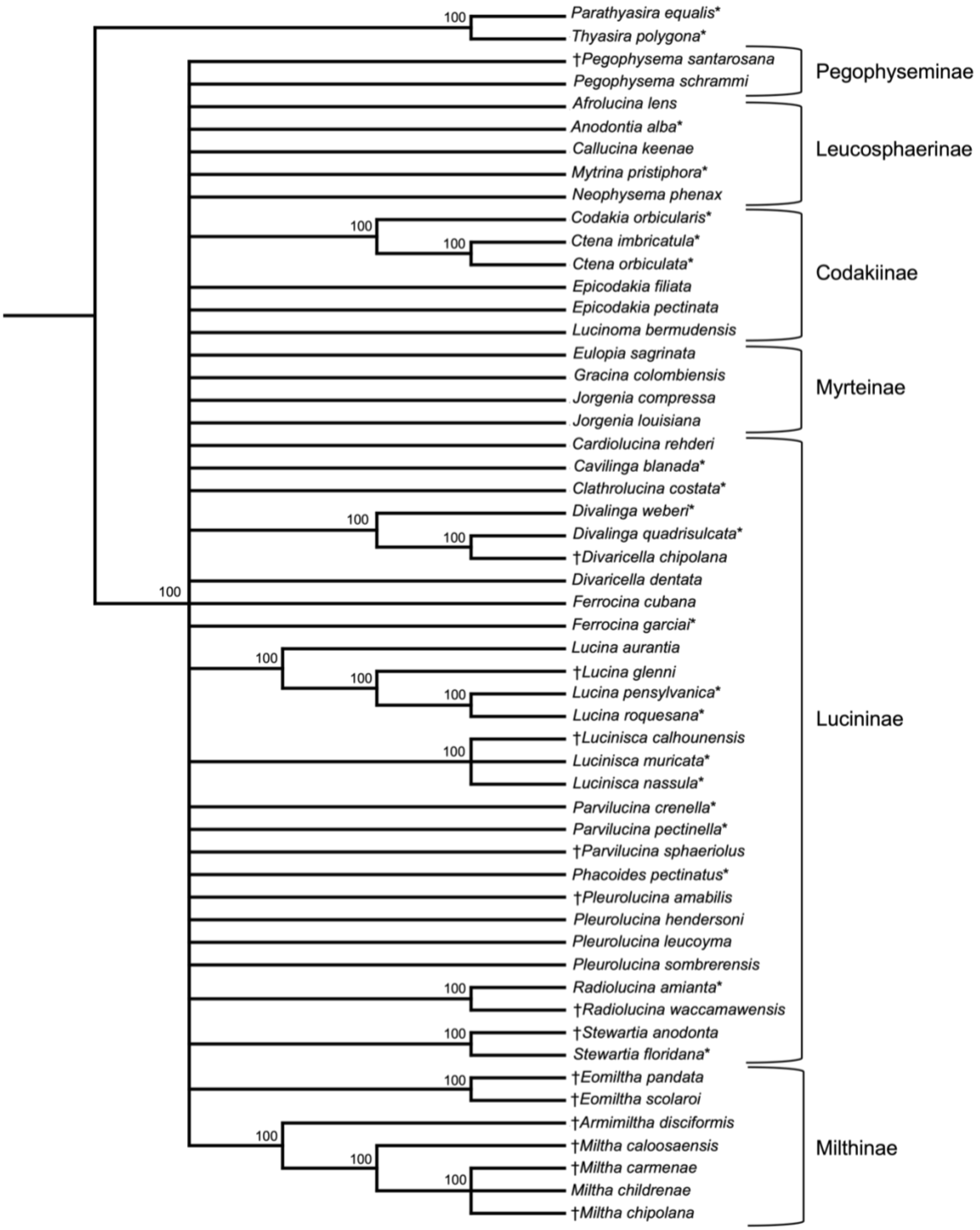

Figure 4: Combined morphological and molecular (concatenated dataset of 18S rRNA, 28S rRNA, and cyt *b*) cladogram of extant and fossil taxa produced using parsimony analysis. Support values at nodes are bootstrap. Subfamilies designated in Taylor et al. (2011; 2016). Asterisks indicate molecular data is available for that species; dagger indicates that a species is extinct.

Molecular Only

When analyzing only the molecular data (Figure 5A), several differences emerge between the present study and those of Taylor et al. (2011, 2016), despite using a similar dataset. Firstly, this study includes fewer taxa, which likely contributes to differences in results. Secondly, the alignment was performed separately using Taylor et al.'s parameters, and gap removal was handled independently using the same parameters, resulting in different sequence lengths after processing with Gblocks. While the sequence lengths are comparable considering the smaller dataset in this study, the differences are still notable. Lastly, although this study used the same Bayesian settings as Williams and Taylor (2011), Taylor et al. (2016) discarded 10% of the first trees, as opposed to the 25% discarded in this analysis, which may also contribute to the variation in molecular results.

Comparative Analysis of Morphological, Molecular, and Combined Data

To streamline the comparison of tree topologies, taxa unique to either this study (Figures 1 and 3) or to the combined gene tree in Taylor et al. (2016) (Figures 1, 2, and 3) were pruned. The resulting pruned trees differed in both resolution and congruence from each other (Figure 5). The re-analyzed molecular only tree had 17 nodes (Figure 5A), whereas the pruned tree from Taylor et al. (2016) had 18 nodes, with nodes distributed across taxonomic levels (Figure 5B). The pruned combined morphological-molecular tree had eight nodes (Figure 5C), and the pruned morphology only tree had four nodes (Figure 5D). The morphological-only phylogenetic tree (Figure 5D) displayed three clades that were congruent and one clade that was not congruent (*Clathrolucina costata* and *Cavilinga blanada*) to the published molecular phylogeny (Figure 5B). The addition of molecular data to the morphological shell character dataset produced a phylogenetic tree (Figure 5C) that had more resolution than the morphological only tree (Figure

5D). The combined tree (Figure 5C) removed *Clathrolucina costata* and placed *Cavilinga blanada* near *Lucina roquesana* and *Lucina pensylvanica*, which was also congruent to the published molecular phylogeny (Figure 5B). The combined dataset resulted in more resolution and congruent clades for the placement of *Codakia orbicularis* with *Ctena* spp. and the resolution of a clade for *Parvilucina crenella* and *Parvilucina pectinella* (Figure 5C). Only the relationships between the *Divalinga* clade and the *Cavilinga*/*Lucina* clade were incongruent for the combined (Figure 5C) and the molecular only (Figure 5B) trees.

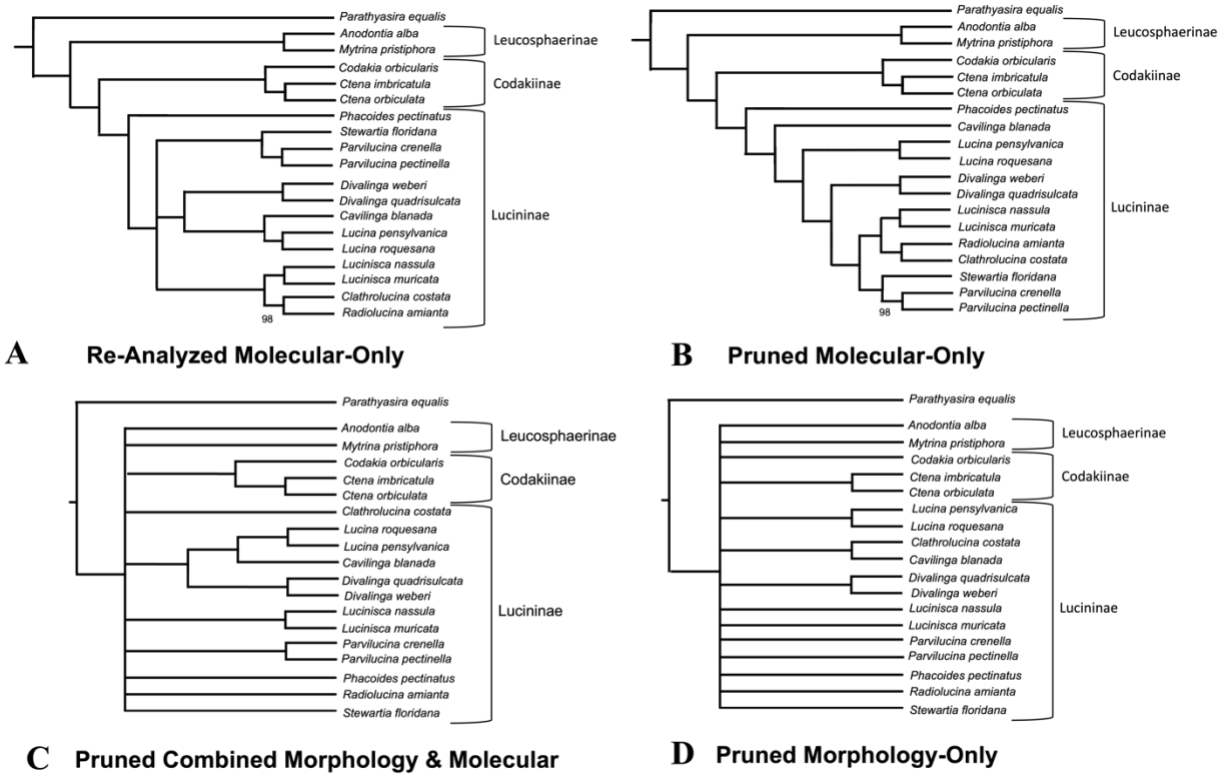

Figure 5: Bayesian cladograms of lucinid taxa for: **A** molecular-only using 18S rRNA, 28S rRNA, and cyt *b* from this study; **B** molecular-only using 18S rRNA, 28S rRNA, and cyt *b* pruned from (Taylor et al. 2016); **C** combined morphological and molecular pruned from Figure 1.3; and **D** morpholgy-only pruned from Figure 1.1. Subfamilies designated in Taylor et al. (2011; 2016).

Discussion

The phylogenetic analyses presented here provide critical insights into the evolutionary relationships within the family Lucinidae by leveraging both morphological and molecular datasets. This study highlights the advantages and limitations of two widely used methods—parsimony and Bayesian inference—for reconstructing phylogenetic trees, ultimately demonstrating the superior resolution and support offered by Bayesian approaches for these data. These findings underscore the importance of integrating molecular data with traditional morphological characteristics, which not only enhances the resolution of phylogenetic relationships but also aligns with broader efforts to refine lucinid taxonomy. In this discussion, we evaluate the implications of these results for lucinid classifications, explore the influence of molecular data on phylogenetic resolution, and propose taxonomic reassignments informed by these analyses. This study determined that shell characters in lucinids do contain phylogenetic signal, although single character states could not be used to define nodes. Nonetheless, we have developed a suite of shell morphologic characters for incorporation into lucinid Bayesian molecular phylogenetic analyses to integrate fossil taxa into current lucinid taxonomies. Shell characteristics are meaningful characters.

Comparison of Phylogenetic Methods

In this study, phylogenetic trees generated using parsimony and Bayesian inference were compared to assess their suitability for reconstructing lucinid phylogenetic relationships (Figures 1–4). Bayesian trees (Figures 1 and 3) and parsimony trees (Figures 2 and 4) differed in resolution and node support. For both morphology-only (Figures 1 and 2) and combined morphological-molecular analyses (Figures 3 and 4), Bayesian methods generally provided greater resolution. Morphological characters may evolve rapidly or with rate heterogeneity,

making Bayesian methods particularly valuable when analyzing relatively few characters, as noted in other studies (e.g., Wright and Hillis 2014). While parsimony with implied weighting can sometimes yield higher-resolution trees than Bayesian methods (Smith 2019), we preferred Bayesian analyses for lucinid datasets because they allow for different evolutionary models to be applied to morphological and molecular data.

Having 100% bootstrap support for both the morphology-only (Figure 2) and combined molecular and morphological (Figure 4) cladograms is unusual and warrants further scrutiny. Typically, achieving 100% bootstrap support across all nodes is rare because phylogenetic analyses often deal with uncertain evolutionary relationships, and bootstrap values reflect the robustness of these relationships. Morphological data, in particular, are prone to ambiguity due to homoplasy and missing data, which usually results in lower bootstrap support. When molecular and morphological data are combined, one might expect stronger support for some nodes, but conflicts between the datasets often lead to lower support values for others. Therefore, the presence of 100% bootstrap support in both the morphology-only and combined analyses suggests either an unusually high level of certainty in the tree's structure or a potential overfitting of the data. This high support could indicate a lack of variability in the dataset or may reflect issues with overfitting, where a small or uninformative dataset yields perfect support. As such, it is essential to assess the methodology and the quality of the data to ensure that the analysis accurately captures the evolutionary relationships without overestimating the reliability of the tree. Consequently, the remainder of this discussion focuses on phylogenies generated by Bayesian methods.

For all cladograms, there are a lot of branches with no support, meaning they should be collapsed (Felsenstein 1985; Hillis and Bull 1993). If you collapse those branches and the

topology doesn't change, then that is support for the placement (Müller 2004; Eckert et al. 2013). This approach reflects the idea that when weakly supported branches do not alter the overall tree structure, the phylogenetic placement is considered more robust. Therefore, collapsing such unsupported branches helps to focus on the more reliable aspects of the phylogeny while avoiding over-interpretation of unsupported relationships.

Impact of Molecular Data on Phylogenetic Resolution

In general, we found that subfamily placement had higher resolution for the combined morphologic and molecular tree (Figure 3) than for morphology-only tree (Figure 1) and although similar, do show some incongruence. Studies suggest that molecular data should not automatically be considered more accurate when morphological and molecular datasets are incongruent (Pisani et al. 2007; Scotland et al. 2003; Wiens 2004). Furthermore, the incorporation of molecular data into combined analyses often increases tree resolution without reducing congruence, enhancing the phylogenetic signal compared to morphology-only analyses (Wiens 1998; Lee and Worthy 2011). Further, the tree produced using both morphological and molecular data had higher resolution and congruence to the published tree of Taylor et al. (2016) than the morphological-only tree (Figures 5B, 5C, and 5D). This implies that the addition of molecular data may produce higher resolution and better congruence compared to morphological data only. Molcular phylogenies should also be used with morphological characters, if possible, when incorporating fossil taxa into phylogenetic analyses (O'Reilly et al. 2016).

Direct comparison of our work to the most recent published lucinid molecular phylogenies (Taylor et al. 2016, Figure 1, 2, and 3) indicates that morphological phylogenies and combined morphologic and molecular phylogenies are less resolved (Figure 5). Although the resolution is lower, the pruned morphological-only tree (Figure 5D) and the combined

morphological and molecular phylogeny (Figure 5C) reveal some relationships consistent with Taylor et al. (2016) and the reanalyzed molecular data from that study (Figure 5B). Further, in a study evaluating lucinid evolutionary history loss in the Western Atlantic since the Pliocene, a morphological dataset derived from Bretsky (1970, 1976) was combined with published 18S rRNA gene molecular data, which resulted in a tree topology similar to the Taylor et al. (2011) 18S rRNA gene molecular phylogenetic tree (Christie et al. 2016; Christie 2017). However, a direct comparison of our results to those of Christie (2017) is not possible because the tree did not have species labels listed on branch tips.

Our results indicate that data type (morphological or molecular, gene-specific) and taxa (sample size and diversity represented) impact resulting tree topologies (Figure 5A). The resulting Bayesian trees have low bootstrap values. The data used, including specific genes sequences or gene combinations, influenced resulting tree topology, as exemplified by the topology differences between each single gene trees (18S rRNA, 28S rRNA, and cyt *b*), as well as the combined gene tree in Taylor et al. (2016). Here, we analyzed a molecular dataset with only 18 ingroup taxa and found that it is less resolved than trees that sampled more taxa (Figure 5A vs. Figure 5D). This finding agrees with other studies that found the number of taxa number and inclusion of particular taxa affect topology. However, a distinction between resolution and congruence must be made, as Christie (2017) found congruence, although at lower resolution, with a Taylor et al. (2011) molecular phylogenetic tree, despite a limited sample size for taxa with molecular data (n = 10). Fewer taxa in a phylogenetic analysis often result in reduced resolution, increased uncertainty, and potential biases in tree topology (Wiley and Lieberman 2011). With fewer taxa, there is a greater risk of overlooking evolutionary relationships or misinterpreting shared derived characters, which can impact the interpretation of both branch

support and tree topology (Baker and DeSalle 1997). Furthermore, the reduced number of taxa can also increase the sensitivity of the analysis to issues like missing data or the inclusion of problematic taxa, which can cause different tree topologies or altered branch lengths (Rokas et al. 2003).

Comparisons with Lucinid Classifications

Our phylogenetic results that included morphologic characters result in trees that are not concordant with the Chavan's (1969) classification or Bretsky's (1970, 1976) phenetic (numerical taxonomy) phylogeny of lucinids, but are in agreement with published molecular phylogenies, albeit with lower resolution (Williams et al. 2004; Taylor et al. 2011, 2014, 2016); Christie 2017). Chavan (1969), who developed a classification for Lucinidae without employing a phylogenetic analysis, proposed a taxonomy that is not supported by the results of this study (Figures 1 – 4). Bretsky's (1970) correlation and distance phenograms (Figures 3 and 4) are highly resolved and were comparable to our phylogeny at the generic level but are not useful at the subfamily level and do not resemble either the Bayesian-inferred (Figure 1) or parsimony-based (Figure 2) morphological phylogenies produced here. Nonetheless, the morphologic phylogenies presented here, and in combination with the phenetic classifications presented by Bretsky (1970), demonstrated that morphologic characters were useful at species- and genus-scale taxonomic scales, but become increasingly confounded at higher taxonomic levels. Further, a classification of the lucinid genus *Anodontia* found that 25 species used in a molecular phylogeny could be distinguished based on morphological data including shell (size, shape, sculpture, periostracum, color, ligament, hinge, anterior adductor muscle scars, lunule, pallial line, and secondary pallial attachment scars) and soft anatomical (mantle gills) characters,

445 although morphologic data were not included in their phylogenetic analyses (Taylor and Glover
446 2005).

447 Molecular phylogenies of lucinids have changed over time, owing to differences in data
448 and methods used for analyses (Williams et al. 2004; Taylor et al. 2011, 2014, 2016; Christie
449 2017). One of the first molecular analyses of Lucinidae found monophyly for the family and
450 identified several clades within the family with high support (Williams et al. 2004). Their results,
451 however, showed major incongruence with older morphology-based classifications (Chavan
452 1969; Bretsky 1970, 1976), indicating that a revision to the family was needed (Williams et al.
453 2004). Following this, Taylor et al. (2011) presented a molecular phylogeny for extant taxa,
454 which supported seven subfamilies, with four previously established subfamilies (Codakiinae,
455 Lucininae, Fimbriinae, and Myrteinae) and three new subfamilies (Pegophyseminae,
456 Leucosphaerinae, and Monitilorinae), but taxa belonging to the unconfirmed subfamily Milthinae
457 (*Miltha* and *Eomiltha*) were missing from the analysis. The most recent molecular phylogenetic
458 analyses have focused on taxa from specific locations, such as deep water (2,000 m) habitats
459 (Taylor et al. 2014) or geographic regions such as the Western Atlantic (Taylor et al. 2016), and
460 how those taxa are placed within the seven subfamilies established in Taylor et al. (2011).

461 Modern Reassignments and Alternative Taxonomies

462 The combined morphological and molecular analyses outlined in this study provided a
463 means to assess at least two existing taxonomic assignments: *Divaricella chipolana* and
464 *Armimiltha disconformis*. Based on evidence outlined below, we suggest that a reexamination of
465 and a classification change of *Divaricella chipolana* to *Divalinga chipolana* and *Armimiltha*
466 *disconformis* to *Miltha disconformis* may be necessary.

Subsequently, most extant genera originally placed in Milthinae have been reassigned to *Anodontia* and *Pegophyesema* based on molecular data (Taylor et al. 2011). At present, the Milthinae includes 4 genera: *Armimiltha* (extinct), *Eomiltha* (extinct), *Miltha,* and *Retrolucina*, with no published molecular data currently available for the extant species (Taylor et al. 2011; 2016). However, based on our results, we consider *Armimiltha* to be a potential junior synonym of *Miltha*.

Our analyses found that the Miocene species *Divaricella chipolana* was part of a subclade that includes two extant *Divalinga* species, instead of the extant *Divaricella dentata* for all morphology-only and combined morphological and molecular phylogenies (Figures 1 – 4). The Divaricellinae subfamily was proposed for all lucinids with divaricate ribs by Gilbert and van de Poel (1967) and was used by Chavan (1969) to describe convex, rounded shells with divaricate or undulating external sculpture. Chavan (1969) assigned 11 genera and subgenera to the subfamily, including *Divaricella* and *Divalinga*. The classification by Bretsky (1976) differs from that of Chavan (1969) by defining *Divalinga* as a subgenera of *Divaricella*. Further, Bretsky (1976) considered *Divaricella chipolona* similar to *Divaricella* (*Divalinga*) *quadrisculata*. Molecular phylogenies indicate taxa with divaricate sculpture belonging to Divaricellinae (Chavan 1969), including *Divaricella* and *Divalinga*, are not closely related and reveal differences in morphology including rib construction, hinge, and ligament (Taylor et al. 2011, 2016). Further, Chavan (1951) restricts *Divaricella* to species with absent or obsolete lateral teeth, and *Divalinga* to species with well-developed lateral teeth. The specimens of *Divaricella chipolona* examined in this study had well-developed lateral teeth, indicating that it should be reassigned to *Divalinga*. Notably, neither of the *Divaricella* taxa (extinct *D. chipolana*

and extant *D. dentata*) included in this study were represented in the molecular dataset, whereas both *Divalinga* species were.

The other highly resolved groups within other subfamilies, such as Codakiinae and Lucininae, had combinations of extant and extinct taxa with and without molecular data in our study.

The addition of fossil taxa, even without molecular data from extant members of the subfamily, contributed to a high resolution within the Milthinae. For example, *Armimiltha disconformis* was grouped with four species belonging to *Miltha* in morphological parsimony and Bayesian analyses (Figures 1, 2, and 4). However, in the combined Bayesian tree (Figure 3), this placement was unresolved, likely because these taxa contributed only morphological data. Other studies have shown that including fossils can increase the number of resolved nodes in phylogenetic analyses (Koch et al. 2021), underscoring their importance in elucidating evolutionary relationships.

The taxonomic history of *A. disconformis* (Heilprin 1886) llustrates the complexity of Milthinae classification. Initially assigned to *Lucina*, the species was later transferred to *Miltha* by Gardner (1926) and Mansfield (1937). Olsson and Harbison (1953) introduced the subgenus *Armimiltha* within Phacoides and designated *P. (A.) disconformis* as the type species. These shifts reflect evolving interpretations of shell morphology and phylogenetic relationships.

Chavan (1969) further refined the taxonomy of Milthinae by defining it as a subfamily based on distinct morphological traits, such as a solid, compressed shell, a long anterior adductor muscle scar, and faint concentric sculpture. Chavan assigned 22 genera and subgenera to Milthinae, including *Miltha*, *Gibbolucina* (*Eomiltha*), *Pegophyesema*, and *Anodontia*. Within this

framework, *Saxolucina* (*Armimiltha*) was considered a junior synonym of *Saxolucina* (*Plastomiltha*).

Bretsky (1976) later reassigned *Armimiltha* as a subgenus of *Miltha*, grouping it alongside other subgenera, including *Eomiltha*, *Plastomiltha*, and *Lucinoma*. These revisions reflect ongoing debates in the taxonomy of Milthinae and highlight the challenge of integrating fossil data into phylogenetic frameworks. While molecular data would strengthen these analyses, a morphology-based taxonomy remains feasible, particularly with comprehensive fossil character suites. Future work could refine these classifications and propose alternative taxonomies, leveraging combined morphological and molecular datasets when available.

Integrating Morphological and Molecular Data

Integrating morphologic and molecular data is essential for robust lucinid phylogenetic analyses, as molecular data enhances tree resolution while morphologic data enables the inclusion of fossil taxa (Figures 1–4). Similarly, published studies demonstrate increased accuracy when combining morphological and molecular characters, as this approach integrates complementary datasets to resolve phylogenetic relationships more robustly (Wiens 2009; Gatesy et al. 2003; Lee and Worthy 2012). This integration is particularly valuable for incorporating fossil taxa that lack molecular data, thereby enhancing phylogenetic inference across broader temporal scales (Donoghue et al. 1989; O'Leary et al. 2013). Combined analyses offer a comprehensive perspective on taxonomic relationships by integrating molecular and morphological data. This approach leverages the strengths of both methods and extends the temporal scope by incorporating valuable insights from evolutionary history.

Conclusions

For the first time, a lucinid morphological phylogeny that directly combined results with molecular gene phylogenies are presented. Comparisons between multiple phylogenetic inference methods indicated that parsimony and Bayesian analyses resulted in similar topologies, and that parsimony can (but not always) be less resolved. Morphological phylogenies had low resolution with numerous polytomies at the subfamily level and morphological characters seemed to have more phylogenetic signal at the genus level (e.g., *Miltha*, *Ctena*, *Stewartia*, *Ferrocina*, *Pleurolucina*, *Lucina*, *Radiolucina*, *Eomiltha*, and *Epicodakia*). Combinations of morphological and molecular data produce phylogenies were less resolved than molecular-only analyses but were still useful for assigning fossil taxa to genera. Since no character states were diagnostic for any specific clade, we propose a character suite for use in combined morphological and molecular phylogenies. This approach demonstrates strong congruence despite relatively low resolution and serves as a proof of concept for incorporating fossil taxa into statistically supported phylogenetic analyses.

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